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Enzyme-linked Immunosorbant Assays for Identification of Biological Agents in Sample Unknowns: NATO SIBCA Exercise V

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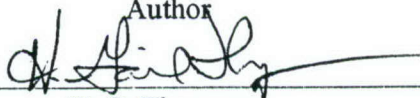
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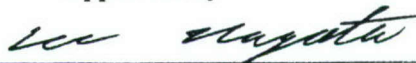
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Abstract

In March 2004, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the fifth international training exercise on identification of biological agents. Seventeen NATO/PfP national laboratories participated: Austria, Bulgaria, Canada, Denmark, France, Germany (2 laboratories), Hungary, Italy, The Netherlands, Norway, Poland, Spain, Sweden, Switzerland, the United Kingdom, and the United States. The designated laboratory for Canada was Defence R&D Canada – Suffield (DRDC Suffield). Participant laboratories were sent eight leaves on which sample unknowns had been adsorbed. Participants were advised that samples would contain any one of the following gamma-irradiated organisms: *Bacillus anthracis*, *Yersinia pestis*, *Brucella melitensis*, *Francisella tularensis*, *Vibrio cholerae*, *Burkholderia mallei*, Venezuelan equine encephalitis (VEE) virus, vaccinia virus, *Coxiella burnetii*, or yellow fever virus. An immunological-based technology, the enzyme-linked immunosorbant assay (ELISA), was used at DRDC Suffield for screening of sample unknowns. Antigen capture ELISAs for each of the 10 possible biological agents were used to screen the samples. Five biological agent unknowns were identified by ELISA, three at the species level: *V. cholerae*, *F. tularensis*, and *Y. pestis*, and two at the genus level: both *Brucella spp.* No agents were identified in three additional samples. A comparison of the ELISA results with the identity of organisms in SIBCA sample unknowns, as revealed by Dugway Proving Ground following the exercise, indicated confirmed identification of four of the agents and the sample blank, one unconfirmed identification, and two false negative identifications.

Résumé

En mars 2004, le sous-groupe du Panel VII d'échantillonnage et d'identification des agents biologiques (SIBCA) de l'OTAN a conduit le cinquième exercice international de formation à l'identification d'agents biologiques. Dix-sept laboratoires nationaux du Partenariat pour la paix de l'OTAN ont participé : L'Autriche, la Bulgarie, le Canada, le Danemark, la France, l'Allemagne (2 laboratoires), la Hongrie, l'Italie, les Pays-Bas, la Norvège, la Pologne, l'Espagne, la Suède, la Suisse, la Grande-Bretagne et les États-Unis. Recherche et Développement pour la défense Canada – Suffield était le laboratoire désigné pour représenter le Canada. Les laboratoires participants ont reçu huit feuilles minces sur lesquelles des échantillons inconnus avaient été adsorbés. Les participants ont été avisés que les échantillons pouvaient contenir l'un des organismes irradié par des rayons gamma *Bacillus anthracis*, *Yersinia pestis*, *Brucella melitensis*, *Francisella tularensis*, *Vibrio cholerae*, *Burkholderia mallei*, le virus de l'encéphalomyélite équine du Venezuela (EEV), le virus de la vaccine, *Coxiella burnetii* ou le virus de la fièvre jaune. DRDC Suffield a utilisé une technologie basée sur l'immunologie, le dosage immunoenzymatique (ELISA) pour analyser les échantillons inconnus. Des ELISA à capture d'antigènes pour chacun des 10 agents biologiques potentiels ont été utilisées pour analyser les échantillons. Cinq agents biologiques inconnus ont été identifiés par ELISA dont trois au niveau de l'espèce : *V. cholerae*, *F. tularensis* et *Y. pestis* et deux au niveau du genre : tous les deux *Brucella spp.* Aucun agent n'a été identifié dans les trois échantillons restants. Une comparaison des résultats ELISA avec l'identité des organismes dans les échantillons inconnus SIBCA, tels que révélés après les exercices par Dugway Proving Ground, ont confirmé l'identification de quatre des agents et de l'échantillon blanc et indiqué une identification non confirmée et deux identifications de faux négatifs.

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Executive summary

Introduction: NATO/Partner for Peace (PfP) Forces may be required to support battlefield or peacekeeping operations in areas of the world where biological weapons have been used, or where there is a threat of their use. Under such circumstances, samples known to, or suspected of, containing biological agents, are likely to be referred to NATO/PfP laboratories for identification or confirmation of biological agent content. To evaluate the capabilities of NATO/PfP laboratories in identifying biological agents in samples, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) have sponsored a number of international training exercises in which participant laboratories have been asked to identify agents in sample unknowns.

In March 2004, SIBCA conducted the fifth international training exercise on identification of biological agents. Seventeen NATO/PfP national laboratories participated: Austria, Bulgaria, Canada, Denmark, France, Germany (2 laboratories), Hungary, Italy, The Netherlands, Norway, Poland, Spain, Sweden, Switzerland, the United Kingdom, and the United States. Participant laboratories were sent leaves on which sample unknowns had been adsorbed. The participating laboratory for Canada was Defence R&D Canada – Suffield (DRDC Suffield). DRDC Suffield used several different technologies to screen the SIBCA samples, one of which was the enzyme-linked immunosorbant assay (ELISA). This report describes the results obtained in screening SIBCA samples for 10 different biological agents by ELISA.

Results: Antigen-capture ELISAs for *Bacillus anthracis*, *Coxiella burnetii*, *Yersinia pestis*, *Francisella tularensis*, *Vibrio cholerae*, *Brucella melitensis*, VEE virus, *Burkholderia mallei*, Vaccinia virus, and Yellow fever virus were used to screen SIBCA samples for homologous agents. Five biological agent unknowns were identified by ELISA, three at the species level: *V. cholerae*, *F. tularensis*, and *Y. pestis*, and two at the genus level: both *Brucella* spp. Two samples containing *C. burnetii* and vaccinia produced false negative reactions. One sample was correctly identified as a blank.

Significance of results: The results of this report demonstrate that the ELISA is a useful tool for identification of biological unknowns. In addition, successful removal of biologicals from the surfaces of foliage represents an expansion of DRDC Suffield's capabilities to isolate and identify agents from a variety of types of matrices. Participation of DRDC Suffield in NATO/PfP SIBCA training exercises is valuable, as it provides a means for DRDC Suffield to measure and evaluate, against international standards, in-house capabilities in the identification of biological agents from sample unknowns in a variety of matrices.

Future goals: Three of the 10 agent ELISAs used in this exercise had not been optimized for sensitivity, and most had not been evaluated for reactivity with common battlefield materials. Further work is required to complete the optimization of all 10 agent ELISAs, to complete the screening of agent ELISAs against a standard panel of agents and potential interferents, and to assess ELISAs for assay reproducibility on a statistical basis. In addition, incorporation of monoclonal antibodies into developed ELISAs would result in greater long-

term assay reproducibility, as well as enhanced specificity for a number of assays in which species-specific identification would be an asset e.g., *Brucella spp.* Furthermore, each agent ELISA should be tested with live agent, to confirm assay sensitivity with live materials. In the longer term, investigation of alternative, more sensitive immunological techniques for identification of biological agents is desirable, as is the development of multiplexed immunological assay systems for the simultaneous screening of samples for multiple agents.

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Sommaire

Introduction : Les Forces du partenariat pour la Paix (PPP) de l'OTAN peuvent être requises de soutenir des opérations en champs de bataille ou de maintien de la paix dans des territoires du monde où les armes biologiques ont été utilisées ou bien quand ces dernières représentent une menace. Dans de telles circonstances, des échantillons connus ou suspects de contenir des agents biologiques seraient vraisemblablement référés aux laboratoires du PPP de l'OTAN pour l'identification ou la confirmation du contenu biologique de ces agents. Pour évaluer les capacités des laboratoires du PPP de l'OTAN à identifier des agents biologiques dans les échantillons, le Sous-groupe du Panel VII d'échantillonnage et d'identification des agents biologiques et chimiques (SIBCA) a parrainé un certain nombre d'exercices internationaux de formation durant lesquels les laboratoires participants ont été requis d'identifier des échantillons inconnus.

En mars 2004, SIBCA a conduit le cinquième exercice international de formation concernant l'identification d'agents biologiques. Dix-sept laboratoires nationaux du Partenariat pour la paix de l'OTAN ont participé : L'Autriche, la Bulgarie, le Canada, le Danemark, la France, l'Allemagne (2 laboratoires), la Hongrie, l'Italie, les Pays-Bas, la Norvège, la Pologne, l'Espagne, la Suède, la Suisse, la Grande-Bretagne et les États-Unis. Les laboratoires participants ont reçu huit feuilles minces sur lesquelles des échantillons inconnus avaient été adsorbés. Recherche et Développement pour la défense Canada – Suffield (DRDC Suffield) était le laboratoire qui représentait le Canada. DRDC Suffield a utilisé plusieurs technologies différentes pour analyser des échantillons SIBCA dont l'une d'elles était le dosage immunoenzymatique (ELISA). Ce rapport décrit les résultats obtenus de l'analyse des échantillons SIBCA de dix différents agents biologiques par ELISA.

Résultats : Des ELISA à capture d'antigènes pour *Bacillus anthracis*, *Coxiella burnetii*, *Yersinia pestis*, *Francisella tularensis*, *Vibrio cholerae*, *Brucella melitensis*, le virus EEV, *Burkholderia mallei*, le virus de la vaccine et le virus de la fièvre jaune ont été utilisées pour analyser les échantillons SIBCA pour les agents homologues. Cinq agents biologiques inconnus ont été identifiés par ELISA dont trois au niveau de l'espèce : *V. cholerae*, *F. tularensis* et *Y. pestis* et deux au niveau du genre : tous les deux *Brucella spp.* Deux échantillons contenant *C. burnetii* et la vaccine ont produit des réactions de faux négatifs. Un échantillon a été correctement identifié comme blanc.

La portée des résultats : Les résultats de ce rapport indiquent que ELISA est un outil utile pour identifier les inconnus biologiques. De plus, le fait de réussir à enlever les agents biologiques des surfaces d'un feuillage représente une expansion des capacités de RDDC Suffield concernant l'isolation et l'identification des agents à partir d'une variété de types de matrices. La participation de RDDC Suffield aux exercices de formation SIBCA du PPP de l'OTAN est précieuse puisqu'elle procure à DRDC Suffield un moyen de mesurer et d'évaluer les capacités internes d'identification d'agents biologiques, à partir d'échantillons inconnus dans une variété de matrices, par rapport aux normes internationales.

Les buts futurs : Trois des ELISA des 10 agents utilisés dans cet exercice n'avaient pas été optimisées pour la sensibilité et la plupart n'avaient pas été évaluées pour la réactivité avec les matériaux communs sur les champs de bataille. De plus amples travaux sont requis pour compléter l'optimisation des ELISA de 10 agents, pour compléter l'analyse ELISA des agents par rapport à un panel normatif d'agents et d'interférents potentiels et pour évaluer ELISA au sujet de la reproductibilité des biotests, sur une base statistique. De plus, l'incorporation d'anticorps monoclonaux dans des ELISA développées résulterait en une reproductibilité des biotests à plus long terme, tout en améliorant la spécificité pour un certain nombre de biotests pour lesquels l'identification spécifique à une espèce serait un avantage comme *Brucella spp* par ex. De plus, chaque ELISA d'agent devrait être testée avec un agent vivant pour confirmer la sensibilité du biotest aux matériaux vivants. À plus long terme, il serait souhaitable d'étudier des solutions de rechange qui seraient plus sensibles aux techniques immunologiques pour l'identification des agents biologiques, de même que de mettre au point de systèmes de biotests immunologiques multiplexés pour des analyses simultanées d'échantillons de multiples agents.

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Introduction

NATO/Partner for Peace (PfP) forces may be required to carry out military or peacekeeping operations in areas of the world where there is a threat of attack with biological agents, or where the occurrence of biological attack is suspected or confirmed. Under such circumstances, NATO/PfP forces would be expected to take samples of materials suspected of containing biological agents and to forward same to respective national laboratories, where procedures would be carried out to identify the agent unknowns and to confirm their presence in samples. In order to assess national capabilities in the NATO/PfP laboratories for identification of biological agents in samples, the NATO group on Sampling and Identification of Biological and Chemical Agents (SIBCA) organized international training exercises in which participating nations were requested to identify, within a given time period, agents in sample unknowns.

The first SIBCA training exercise for biological agents i.e., SIBCA I, was hosted by Dugway Proving Ground (DPG), UT, in March 1999. Samples, consisting of phosphate buffered saline (PBS) spiked with biological agents, were number coded by DPG, then shipped to participant nations for analysis. Participant nations were advised that biological agents could consist of any one of the following 10 gamma-irradiated organisms: *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae*, Venezuelan Equine Encephalitis (VEE) virus, *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei*, yellow fever virus, vaccinia virus, or *Coxiella burnetii*. The participating laboratory for Canada was Defence Research Establishment Suffield (DRES)¹. DRES screened sample unknowns by two different antibody-based identification technologies, the ThresholdTM device, a light addressable potentiometric sensor (LAPS), and immunochromatographic assays [1, 2]. In addition, a limited analysis by genetic techniques was also used [3].

A second SIBCA training exercise (SIBCA II), again hosted by DPG, was held in February 2000. Six sample unknowns from the list of 10 agents used in the SIBCA I exercise, again suspended in PBS, were sent to the participating laboratories. Two of the samples also contained common battlefield interferents, either burnt vegetation residue or burnt diesel fuel residue. Two technologies were employed by DRES to assess the samples, one genetic-based method [4] and one antibody-based method, enzyme-linked immunosorbent assay (ELISA) [5].

In February 2001, DPG again hosted a SIBCA training exercise (SIBCA III) in which seven samples, six containing agent and one blank, from the same list of 10 inactivated agents as were used in the previous two SIBCA exercises, were sent to each participating laboratory. Three of the samples were agent suspended in soil, while the remainder were in a PBS matrix. DRDC Suffield employed three different technologies for this exercise, including one genetic-based technique [6] and two antibody-based techniques, namely ELISA [7] and ThresholdTM immunoassay [8].

¹ DRES: renamed Defence Research and Development Canada – Suffield (DRDC Suffield) in January, 2001

In January 2002, SIBCA conducted the fourth international training exercise on identification of biological agents. For the fourth SIBCA exercise (SIBCA IV), also hosted by DPG, six swab samples, bearing sample unknowns from the same list of 10 potential agents as previous exercises, were sent to participating countries. DRDC Suffield employed two different technologies for this exercise, one genetic-based technique [9] and an antibody-based technique, namely ELISA [10], complemented by limited Threshold™ assays and bacterial stains techniques.

The fifth international training exercise on identification of biological agents (SIBCA V), again hosted by DPG, was held in March 2004. Seventeen NATO/PfP national laboratories participated: Austria, Bulgaria, Canada, Denmark, France, Germany (2 laboratories), Hungary, Italy, The Netherlands, Norway, Poland, Spain, Sweden, Switzerland, the United Kingdom, and the United States. For the fifth SIBCA exercise, samples received consisted of eight leaves on which agent unknowns, from the same list of 10 potential agents as described for previous exercises, had been adsorbed. Leaves were held inside individual sealed plastic bags until analyses could be performed. Analysis of the SIBCA V sample unknowns by genetic-based technique was initiated in July 2004, while immunological assays were started in October 2004. This report describes the results obtained on screening of SIBCA V sample unknowns by ELISA.

Methods for the analysis of biologicals on leaf surfaces had not been investigated at DRDC Suffield prior to the SIBCA V exercise. However, a survey of literature revealed a number of reports on the surface washing of foliage, particularly in the area of environmental pollutants. Aerosol pollutants on foliage near roadways were reported to have been successfully removed and analysed, including heavy and transitional metals [11, 12, 13, 14, 15, 16] and organics [17]. Other pollutants detected on leaf surfaces near industrial areas included combustion products [17], sulphates and nitrates [18, 19], and polycyclic aromatic hydrocarbons [20, 21]. Biologicals that have been detected on surfaces of leaves included various types of fungi, *E. coli*, and other bacteria such as *Bacillus spp.* and *Coryneform spp.* [22].

In the SIBCA V exercise reported herein, antigen-capture ELISAs were used to analyze sample unknowns for all 10 SIBCA agents. Assays for each agent were configured in such a way that liquid sample unknowns were screened in parallel with homologous agents (positive controls). Five biological agent unknowns were identified by ELISA in SIBCA samples, three at the species level and two at the genus level. A comparison of the ELISA results with the identity of organisms in SIBCA sample unknowns, as revealed by US DPG following the exercise, indicated that four organisms, *V. cholerae*, *F. tularensis*, *Y. pestis*, and *Brucella spp.* had been correctly identified in samples 162, 275, 102, and 573, respectively. Two false negatives, *C. burnetii* (sample 406) and vaccinia (sample 999) were also observed. The identity of sample 804, *Brucella spp.*, was not confirmed, as a sampling error had occurred at DPG (Bruce Harper, DPG, personal communication).

Materials and methods

SIBCA test samples

Pre-exercise information

Eight SIBCA sample unknowns, adsorbed on green leaves (numbered UK102, UK162, UK275, UK406, UK573, UK804, UK810, and UK999)², were received at DRDC Suffield from US DPG on 23 March, 2004. Participants were informed that samples would contain any one of the following killed (cobalt-irradiated) agents: *B. anthracis*, *C. burnetii*, *Y. pestis*, *F. tularensis*, *V. cholerae*, *B. melitensis*, VEE virus, *B. mallei*, vaccinia virus, or yellow fever virus. Ten working days, not necessarily consecutive, were allowed for completion of the analyses, after which time, results were to be forwarded to DPG for collation.

Sample preparation

Post exercise information [30] revealed that each leaf had been spotted with five drops of each agent used (20 µL per drop, 100 µL per leaf) with target concentrations of $10^6 - 10^7$ cfu/leaf for bacteria and $10^7 - 10^8$ pfu/leaf for virus/rickettsia. Biological agents were reconstituted from each leaf as follows. A dry sterile DacronTM-polyester swab (Fisher Scientific, Edmonton, AB) was dipped into a 1.5 mL microfuge tube containing 125 µL PBS, pH 7.4. Once all of the liquid had been absorbed, the wetted swab was moved, with pressure, across one side of the leaf, lengthwise, crosswise, and in circular motions for two minutes. The swab was then placed into an empty, sterile 10 mL tube and capped. The procedure was repeated for the other side of the leaf with a new dry, sterile swab. PBS (0.5 mL, pH 7.4) was added to the tube containing both swabs and the contents vortexed vigorously for one minute. The swabs were squeezed against the side of the tube to expel any remaining liquid and placed into a new tube for storage at -70°C. The PBS containing the sample unknowns for each leaf was aliquoted into four new, sterile tubes, 100 µL each, and stored at -70°C until analyzed. Each leaf was then allowed to air dry, replaced into its original sealing bag, placed into the original freezer box, and stored at -70°C.

Post-exercise identity of sample unknowns

After all laboratories had completed their analyses and the results had been reported, participants were informed by DPG of the identities of agents present in SIBCA sample unknowns. The identities of the agents in the eight sample unknowns received by Canada are presented in Table 1.

² Samples were coded, by mistake, for the laboratory in the UK and sent to DRDC Suffield. However, the DPG exercise coordinator advised DRDC Suffield to continue with the samples received.

Table 1. Agents in SIBCA exercise V samples³

Sample No.	Agent
UK 102	<i>Y. pestis</i>
UK 162	<i>V. cholerae</i>
UK 275	<i>F. tularensis</i>
UK 406	<i>C. burnetii</i>
UK 573	<i>B. melitensis</i>
UK 804	Sampling error ⁴
UK 810	blank
UK 999	vaccinia

Enzyme-linked immunosorbant assays

Antibodies

Unlabelled antibodies

The following antibodies were purchased from commercial sources: *V. cholerae*, polyvalent rabbit antiserum (Inaba, Ogawa), catalogue no. M-2432-50B, lot no. 142625 (Lee Laboratories, Grayson, GA); *V. cholerae* 01, mouse monoclonal IgG, catalogue no. VCM-5261-5, lot no. VC6N11, 0.25 mg/mL (Austral Biologicals, San Ramon, CA); *C. burnetii*, positive control human serum, catalogue no. 4030-02-01, lot no. CBP018 (Integrated Diagnostics, Baltimore, MD); and yellow fever virus, mouse monoclonal ascites (clone 2D12), catalogue no. MAB984, lot no. 20060494 (Chemicon International, Temicula, CA).

Antibody stocks developed under DRES contract by SciLab Consulting Inc. (Redcliff, AB) [23] were as follows: *B. anthracis*, goat IgG, lot no. SC97Ant001, 5 mg/mL, serial no.⁵ CABAC70P0090797; *B. anthracis*, rabbit IgG, lot no. SC97Ant002, 5 mg/mL, serial no. CABAC71P0090797; *Y. pestis*, goat IgG, lot no. SC97YP001, 4 mg/mL, serial no. CAYER3810/08/99;

³ Information provided by US DPG following reporting to DPG of SIBCA V results

⁴ US DPG reported that a sampling error had been made. The sample was to have been *B. anthracis*, but the material deposited on the leaf did not contain *B. anthracis* (Bruce Harper, DPG, personal communication).

⁵ Serial no. assigned by DRDC Suffield MOU database

Y. pestis, rabbit IgG, lot no. SC97YP002, 3 mg/mL, serial no. CAYER9310/08/99; *B. melitensis*, goat IgG, lot no. SC95BM001, 15 mg/mL, serial no. CABru7925/08/9908:52:06; *B. melitensis*, rabbit IgG, lot no. SC97Mel002, 10 mg/mL, serial no. CABru11P0090797; *B. mallei* mallein, goat IgG, lot no. SC97M1001, 10 mg/mL, serial no. 1CaBur21P0090797; *B. mallei* mallein, rabbit IgG, lot no. SC97M1002, 10 mg/mL, serial no. CABur16P0090797; VEE virus, goat IgG, lot no. SC 97VEE002, 4 mg/mL, serial no. CaVen84P0090797; VEE virus, rabbit IgG, 10 mg/mL, lot no. SCVVEE001, serial no. CaVen6P0000529; vaccinia virus, goat IgG, lot no. SC97V001, 5 mg/mL, serial no. CAVac61P0090797; and vaccinia virus, rabbit IgG, lot no. SC97002, 5 mg/mL, serial no. CaVac69P0090797.

The following antibodies were produced under DRES contract by Canadian Bioconcepts Inc. (Saanichton, BC) [24]: *F. tularensis*, bovine IgG, 6.7 mg/mL, serial no. CAFRA5716/08/99; and *F. tularensis*, rabbit IgG, 2.6 mg/mL, serial no. CaFra60P014079.

Coxiella burnetii rabbit IgG, lot no. 030899-01, 5.9 mg/mL and yellow fever rabbit IgG, lot no. 040199-01, 5.0 mg/mL were gifts from the Naval Medical Research Center (NMRC) (Bethesda, MD).

Labelled antibodies

The following horseradish peroxidase-labelled antibodies were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON): rabbit anti-human IgG (whole molecule, lot no. 068H4897); goat anti-rabbit IgG (whole molecule, lot no. 40H8822); and rabbit anti-goat IgG (whole molecule, lot no. 90H8990). Horseradish peroxidase-labelled goat anti-mouse IgG + IgM (H and L chain), lot no. 17011200, was purchased from Caltag Laboratories (Burlingame, CA).

Antibody purification

Vibrio cholerae rabbit antiserum (Lee Laboratories), *C. burnetii* human positive control serum (Integrated Diagnostics), and yellow fever virus mouse ascites (Chemicon International) were purified by a Pierce NabTM protein G spin chromatography kit (Biolynx Inc., Brockville, ON). Procedures followed were those described in the manufacturer's protocol. Antibodies were purified in 100 µL batches and samples were eluted with 400 µL of elution buffer, after which, samples were immediately neutralized by the addition of sodium phosphate, pH 8.0. Fractions containing maximum protein content, typically fraction one, were collected for use in ELISA. Protein concentrations of IgGs resulting from the above described purifications were: *V. cholerae* rabbit IgG: 4.6 mg/mL; *C. burnetii* human IgG: 2.0 mg/mL; and yellow fever virus mouse monoclonal IgG: 2.7 mg/mL.

Antibodies produced by Scilab Consulting Inc. were purified on a Bio-Gel^R Protein G Fast Flow Gel column (Bio-Rad Laboratories, Mississauga, ON) by a High-Performance Liquid Chromatography system (Spectral Physics, San Jose, CA) [9]. Antibodies produced by Canadian Bioconcepts Inc. were purified on a BioRad rProtein ATM column by a Gilson Low Pressure Liquid Chromatography system [24].

Antigens

The following antigens were purchased from commercial sources: Mutacol Berna^R *V. cholerae* live oral vaccine, strain CVD 103-HgR, lot no. 01532401 (Berna Products Corp., Mississauga, ON); stock *V. cholerae* antigen ($\sim 2 \times 10^9$ cfu/mL) was prepared by re-suspending a single vaccine dose in 100 mL Dulbecco A PBS (Oxoid Inc., Nepean, ON); yellow fever virus strain 17D, live attenuated vaccine, lot no. C0229AA (Connaught Laboratories, Willowdale, ON); stock yellow fever virus antigen was prepared by re-suspending a 5 dose vial in 3.0 mL of 0.9% sodium chloride (diluent provided); and *C. burnetii*, Phase 1 antigen, catalogue no. 534P1-V (Vero), lot no. CBPh1-030900WX, formalin inactivated, 1mg/mL (PanBio InDx Inc., Baltimore, MD).

The following cobalt-irradiated antigen stocks were gifts from DPG: *Y. pestis*, India 195/P strain (F1+), 3.6×10^8 cfu/mL; *F. tularensis*, Schu 4 strain, lot no. 95306, 7.8×10^8 cfu/mL; *B. anthracis*, Vollum strain, lot no. 96092, 1.3×10^8 cfu/mL; vaccinia virus, Lister strain, 1×10^7 pfu/mL; and *B. melitensis*, type 2, 4.3×10^9 cfu/mL.

B. mallei mallein complement fixation antigen (ophthalmic), serial no. 91-94, exp. date 95.12.31, was a gift from Animal Diseases Research Institute (Nepean, ON). VEE strain TC83, originally obtained from the US Army Medical Institute of Infectious Diseases, was grown and purified at DRDC Suffield. DRES Run 1, pool 2 (105 µg/mL) was used as antigen stock for the ELISA.

Format

ELISAs for the identification of all 10 SIBCA agents were performed in indirect (antigen capture) assay format. By this method, capture antibody (CAb) is adsorbed to the solid phase and is used to capture the target antigen or sample unknown. Agent ELISAs were used to screen the SIBCA V samples for homologous agents.

Procedures

Agent ELISAs performed on the SIBCA samples included positive and negative (no antigen) controls, and the sample unknowns. To provide sufficient volume for ELISA analysis, the SIBCA samples were diluted 1:20 in PBS prior to use. Samples were assayed two agents per plate, four agents per day. All samples were tested in replicates of three wells.

ELISAs were performed in 96-well NUNC™ Maxisorb microtiter plates purchased from Canadian Life Technologies, Burlington, ON. Positive control antigens and sample unknowns were detected with unlabelled detector antibody (DAb) and indicated by enzyme-labelled indicator antibody (IAb). Washes were performed using a Bio-Tek ELX-50 autostrip washer (Fisher Scientific). Wash steps consisted of five cycles of washing with a volume of 300 µL wash buffer (PBS containing 0.1% bovine serum albumin (Roche Diagnostics, Laval, QC) and 0.1% Tween-20) per wash cycle. Antigen, or sample unknown, DAb, and IAb were diluted, as required, in ELISA buffer (PBS containing 2% BSA and 0.1% Tween-20). Unless otherwise specified, all incubation steps were at 37 °C for 1 hr.

Wells were coated with 100 µL of CAb in coating buffer (carbonate-bicarbonate buffer, 0.05M, pH 9.6) and incubated at 4° C overnight. Plates were washed, then blocked by the addition of 300 µL of blocking buffer (PBS containing 2% BSA), and incubated. Plates were washed, then 100 µL of antigen, or sample unknown, was added, and the plates incubated. Plates were again washed, 100 µL of DAb was added, plates were incubated, then washed. One hundred µL of IAb was added and the plates were incubated, then washed. One hundred µL of substrate solution ((2,2'-azino-di- (3-ethyl-benzthiazoline sulfonate) (ABTS) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD)) was added and the plates incubated at room temperature for 30 min, after which the coloured reaction product was measured in an automated plate reader (see below).

Immunoreagents

The identity and working concentrations of CAbs, DAbs, IAbs, and positive control antigens used in respective agent ELISAs, are shown in Table 2. IAb concentrations were as optimized or as used in previous SIBCA exercises [7, 10].

Table 2. Working concentrations of capture, detector, and indicator antibodies, and positive control antigens used in agent ELISAs

AGENT ELISA	CAb	DAb	IAb (ABTS SUBSTRATE)	POSITIVE CONTROL ANTIGEN
<i>B. anthracis</i> ⁶	goat α - <i>B. anthracis</i> 10 μ g/mL	rabbit α - <i>B. anthracis</i> 15 μ g/mL	goat α -rht-HRP 1:1000	<i>B. anthracis</i> , volum: 1.3x10 ⁷ cfu/mL, 3.25x10 ⁶ cfu/mL
<i>Y. pestis</i> ⁶	goat α - <i>Y. pestis</i> 10 μ g/mL	rabbit α - <i>Y. pestis</i> 25 μ g/mL	goat α -rht-HRP 1:1000	<i>Y. pestis</i> -India (F1+): 3.6x10 ⁶ cfu/mL, 0.9x10 ⁶ cfu/mL
<i>B. melitensis</i> ⁶	goat α - <i>B. melitensis</i> 15 μ g/mL	rabbit α - <i>B. melitensis</i> 20 μ g/mL	goat α -rht-HRP 1:1000	<i>B. melitensis</i> , type 2: 4.3x10 ⁷ cfu/mL, 1.1x10 ⁷ cfu/mL
<i>F. tularensis</i> ⁶	bovine α - <i>F. tularensis</i> 15 μ g/mL	rabbit α - <i>F. tularensis</i> 20 μ g/mL	goat α -rht-HRP 1:3000	<i>F. tularensis</i> , Shu 4: 7.8x10 ⁷ cfu/mL, 1.95x10 ⁷ cfu/mL
<i>V. cholerae</i>	rabbit α - <i>V. cholerae</i> 15 μ g/mL	mouse α - <i>V. cholerae</i> 15 μ g/mL	goat α -ms-HRP 1:3000	<i>V. cholerae</i> vaccine 2-10x10 ⁷ cfu/mL, 0.5-2.5x10 ⁷ cfu/mL
<i>B. mallei</i> ⁶	goat α - <i>B. mallei</i> mallein CF Ag 15 μ g/mL	rabbit α - <i>B. mallei</i> mallein CF Ag 20 μ g/mL	goat α -rht-HRP 1:2000	<i>B. mallei</i> : mallein CF Ag 1:100, 1:400
VEE ⁶	goat α -VEE 10 μ g/mL	rabbit α -VEE 20 μ g/mL	goat α -rht-HRP 1:2000	VEE DRES Run 1, pool 2: 12 μ g/mL, 3 μ g/mL
Vaccinia ⁶	goat α -vaccinia 15 μ g/mL	rabbit α -vaccinia 15 μ g/mL	goat α -rht-HRP 1:2000	Vaccinia, Lister: 0.5x10 ⁷ pfu/mL, 0.125x10 ⁷ pfu/mL
<i>C. burnetii</i>	rabbit α - <i>C. burnetii</i> 15 μ g/mL	human α - <i>C. burnetii</i> 15 μ g/mL	rht α -hu-HRP 1:5000	<i>C. burnetii</i> , Ph 1 50 μ g/mL, 12.5 μ g/mL
Yellow fever	mouse α -yellow fever 15 μ g/mL	rabbit α -yellow fever 20 μ g/mL	goat α -rht-HRP 1:1000	Yellow fever vaccine: 1:2, 1:8

⁶ optimized assay

Data Acquisition, reduction, and analysis

ELISA reaction products were read at A_{405} nm in a Molecular Devices Thermomax^R automated plate reader (Fisher Scientific). Preliminary data analysis was performed using Softmax 3.0 software (Molecular Devices, Menlo Park, CA). Data was then exported to Microsoft Excel 7.0 for reduction and statistical analysis. Reduced data was further exported to Grapher 4 (Golden Software Inc., Golden, CO) for plotting of graphs.

Statistics

Samples were tested in replicates of three wells. Unless otherwise indicated, data points represent the mean of three determinations.

ELISA readings were considered positive if the mean absorbance reading was significantly greater than the background absorbance reading. Statistical significance was established at 20% above the background (no antigen control) absorbance. This reading represents a value of ten times the average CV among replicates. Thus, a signal was considered positive if the value of the sample signal to background signal was 1.2 or greater.

Results

Results obtained by challenge of 10 agent ELISAs with the eight SIBCA sample unknowns, including results of homologous agent positive controls, are summarized in Table 3 and graphically represented in Figures 1-10.

Positive controls for all 10 agent ELISAs (*B. anthracis*, *C. burnetii*, *B. melitensis*, *F. tularensis*, *Y. pestis*, *B. mallei*, vaccinia, VEE, yellow fever, and *V. cholerae*) produced positive reactions in respective homologous assays, thus confirming the validity of each of the agent assays for identification of respective homologous agents. Five SIBCA samples were positive for a single agent in four of the agent ELISAs. Hence, sample 102 was positive in the *Y. pestis* assay, sample 162 was positive in the *V. cholerae* assay, sample 275 was positive in the assay for *F. tularensis*, and samples 573 and 804 were positive in the *B. melitensis* assay.

Table 3. ELISA results: SIBCA V samples

Agent ELISA	Positive Control	SIBCA Sample No.							
		102	162	275	406	573	804	810	999
<i>B. anthracis</i>	+	-	-	-	-	-	-	-	-
<i>Y. pestis</i>	+	+	-	-	-	-	-	-	-
<i>B. melitensis</i>	+	-	-	-	-	+	+	-	-
<i>F. tularensis</i>	+	-	-	+	-	-	-	-	-
<i>V. cholerae</i>	+	-	+	-	-	-	-	-	-
<i>B. mallei</i>	+	-	-	-	-	-	-	-	-
VEE	+	-	-	-	-	-	-	-	-
Vaccinia	+	-	-	-	-	-	-	-	-
<i>C. burnetii</i>	+	-	-	-	-	-	-	-	-
Yellow fever	+	-	-	-	-	-	-	-	-

Agents identified compared with known sample content

Table 4 is a summary of the agents identified by ELISA in the SIBCA V samples, compared with the agent content of samples as revealed by US DPG following the SIBCA exercise.

In four of the eight samples (sample nos. 102, 162, 275, and 573), the agents confirmed present by DPG were correctly identified by ELISA. In addition, the blank, containing PBS only, was correctly identified as negative for agent. Sample 804 was identified as containing *Brucella spp.*, however, this could not be confirmed due to a sampling error in this sample at DPG (Bruce Harper, DPG, personal communication). Two false negative identifications were made, *C. burnetii* in sample 406 and vaccinia virus in sample 999.

Table 4. Agents identified compared with known agent content of SIBCA V samples

SIBCA sample no.	Agent present ⁷	Agent Identified
102	<i>Y. pestis</i>	<i>Y. pestis</i>
162	<i>V. cholerae</i>	<i>V. cholerae</i>
275	<i>F. tularensis</i>	<i>F. tularensis</i>
406	<i>C. burnetii</i>	-
573	<i>Brucella spp.</i>	<i>Brucella spp.</i>
804	Sampling error ⁸	<i>Brucella spp.</i>
810	blank	-
999	vaccinia	-

⁷ Information provided by US DPG following SIBCA exercise

⁸ US DPG reported that a sampling error had been made. The sample was to have been *B. anthracis*, but the material deposited on the leaf did not contain *B. anthracis*.

Discussion

Each of the agent ELISAs used in SIBCA V was challenged with positive controls to ensure the validity of each of the agent assays for identification of respective homologous agents. In previous exercises, a heterologous agent panel, comprising all of the agents in the SIBCA exercise, was also included in each agent ELISA as a means for control for assay specificity [7, 10]. This was considered necessary for these exercises, since most of the assays had not been fully optimized nor had they undergone extensive characterization. However, in the interim, seven of the 10 ELISAs had been optimized. In addition, results of, previous SIBCA exercises have not demonstrated antibody cross-reactivity with any of the heterologous agents in any of the agent assays at the concentrations tested. Therefore, challenge of agent assays with the heterologous agent panel was not considered necessary for this present exercise.

The agents present in SIBCA samples 102, 162, 275, and 573 were correctly identified by ELISA as *B. anthracis*, *V. cholerae*, *F. tularensis*, and *Brucella spp.*, respectively. These results were consistent with those obtained by genetic analyses (Doug Bader, DRDC Suffield, personal communication), thus allowing Canada to report identification of agents in these samples at the NATO Confirmed⁹ level. In addition, a strong signal for *Brucella spp.* was exhibited in both the ELISA and genetic analyses of sample 804, but a sampling error by US DPG did not allow for confirmation of this result. Sample 810 was correctly identified by ELISA as negative, while genetic analyses showed a weak polymerase chain reaction (PCR) signal suggestive of *B. anthracis* (Doug Bader, DRDC Suffield, personal communication). Sample 999, which contained vaccinia virus, was not identified by ELISA, while genetic analysis proposed a NATO Provisional⁹ identification of *Bacillus spp.* (Doug Bader, DRDC Suffield, personal communication).

C. burnetii, identified correctly in sample 406 by genetic methods, was not identified statistically by ELISA. However, as can be seen in Figure 9, there was a strong indication of the presence of *C. burnetii* in sample 406. The signal was actually higher than that of the low positive control (12.5 µg/mL), but did not exceed the statistical positive/negative cut-off of the assay. The problem may have been with the high absorbance of the negative control, which suggests an over-reaction with the substrate, ABTS. The ABTS product literature recommends further dilutions of the primary antibodies and /or conjugate to reduce the intensity of the substrate reaction. However, further investigation of these assay variables at this time was not possible due to depletion of assay reagents. The *C. burnetii* assay was one of the three ELISAs that had not been optimized prior to the exercise and the failure to statistically correctly identify *C. burnetii* in sample 406 highlights the importance of fully optimizing each of the agent assays.

The vaccinia virus in sample 999 was not detected by ELISA. Genetic analyses showed traces of vaccinia in sample 999, however, traces of this virus were similarly seen in most of the samples and were dismissed as contamination. The presence of traces of vaccinia virus by genetic analysis in a number of samples has similarly been reported in previous SIBCA

⁹ NATO SIBCA level of identification [25] where "Confirmed" means identification by two different methods in the presence of a positive control; "Provisional" means identification by one method in the presence of a positive control.

exercises [9]. The ELISA for vaccinia had been optimized prior to the SIBCA V exercise and had a limit of detection (LOD) of 1×10^6 pfu/mL (unpublished result), which is well below the estimated target of $10^7 - 10^8$ pfu/100 μ L applied to the leaf. Therefore, the concentration of vaccinia virus released from the leaf during sample processing may have been less than the LOD of the vaccinia ELISA. Another possibility is that the vaccinia virus, which is four or more times smaller than the bacteria detected on the other leaves [26, 27], may have penetrated the leaf's surface through the cuticular lipophilic pathway. In this case, homogenization of the leaf, followed by analysis of the extract, would be necessary in order to make this material available for detection by ELISA.

Sample 804 produced a strong positive signal for *Brucella* spp., both by ELISA and by genetic analysis. However, DPG reported that a sampling error had occurred with this sample and, although it was intended that the sample contain *B. anthracis*, *B. anthracis* was inadvertently not added. No further information on this sample was forthcoming from DPG and it is unknown, at the time of this report, whether the sample could have mistakenly been spiked with *Brucella* spp. If the sample, indeed, contained a species of *Brucella*, it is not unexpected that the polyclonal antibodies used in the ELISA, which were raised against *B. melitensis*, would be cross-reactive. Cross-reactivity among the main pathogenic *Brucella* spp. (*B. melitensis*, *B. abortus*, and *B. suis*) may be attributed to the O-chain of the smooth lipopolysaccharide (S-LPS). Currently, four types of epitopes on the S-LPS O chain have been described: the M and A epitopes, present on M and A dominant *Brucella* strains, respectively; the common (C) epitope, present on either A or M dominant strains; and the C/Y epitope, common to smooth *Brucella* spp. and *Y. enterocolitica* O:9 [28]. It is also possible that sample 804 was not spiked with *Brucella* spp. by DPG but, rather, that a plant pathogen that cross-reacts with *Brucella* spp., was "washed off" the leaf during sample processing at DRDC Suffield. It is known that *Brucella* spp., although not closely related to any other known animal pathogens, show close genetic relatedness to some plant pathogens and symbionts, including *Agrobacterium*, *Phyllobacterium*, and *Rhizobium* [29]. Further ELISA characterization of the agent in sample 804 by the use of monoclonal antibodies that are specific for the A, M and C (AM) epitopes of *Brucella* spp. may be pursued at DRDC Suffield.

Conclusions

Participation in NATO/PfP SIBCA V has provided DRDC Suffield with the opportunity to assess current capabilities in the immunological identification of biological agent unknowns. In addition, successful removal of biologicals from the surfaces of foliage represents an expansion of DRDC Suffield's capabilities to isolate and identify agents from a variety of types of matrices. Agent ELISAs, in antigen-capture format, were used to screen SIBCA V samples for all 10 possible SIBCA agents. Results indicated that five of the eight samples were correctly identified by ELISA; one blank, three agents at the species level and one agent at the genus level. Agent was identified in one additional sample but the identity of this agent could not be confirmed due to sampling error at the host laboratory. Two additional samples exhibited false negative responses. It is clear from the results of this exercise, that ELISA is a useful tool for identification of biological agent unknowns in environmental samples.

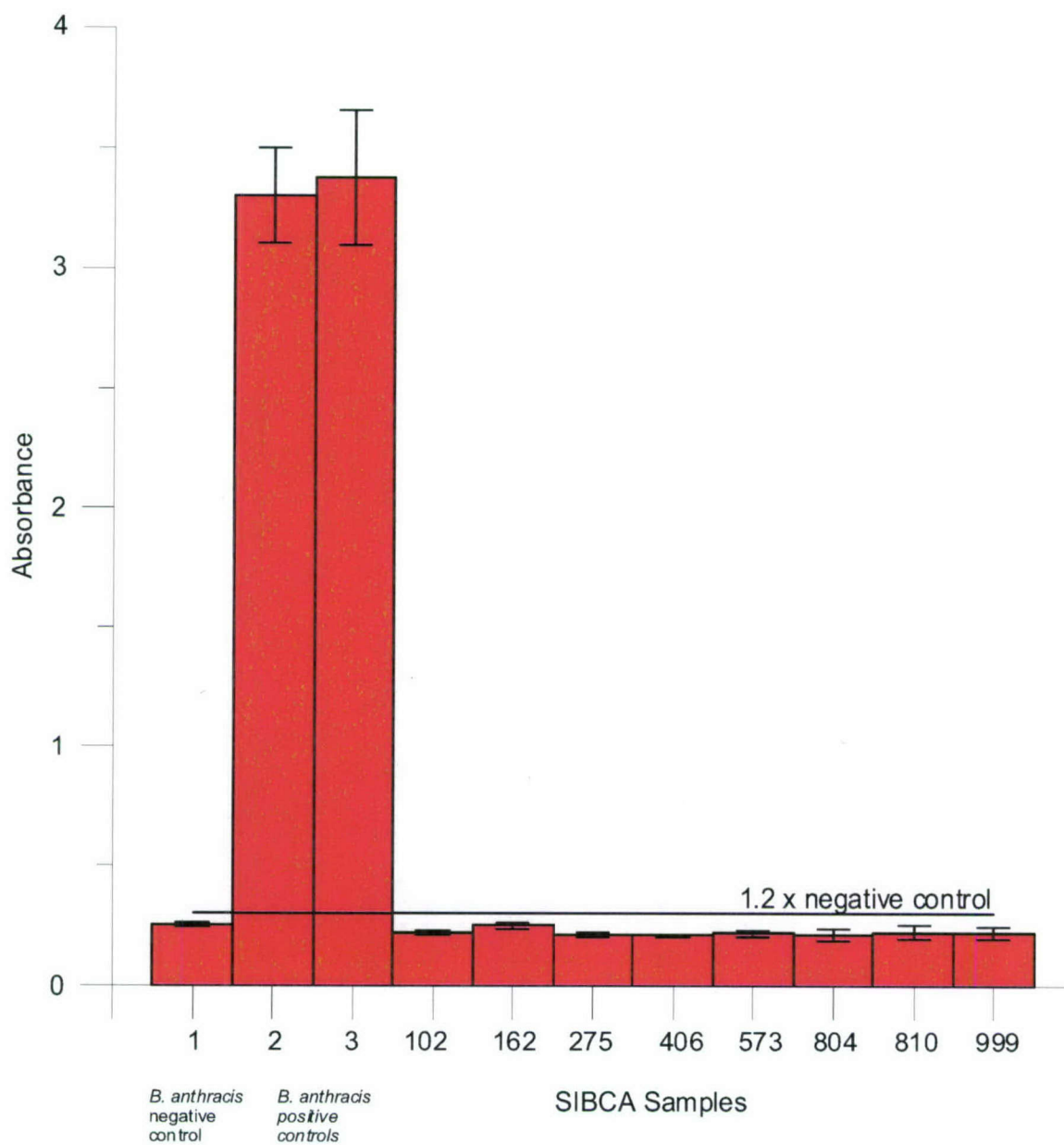


Figure 1. *B. anthracis* ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were 3.25×10^6 and 1.3×10^7 cfu/mL dilutions, respectively. Error bars represent the mean \pm one standard deviation

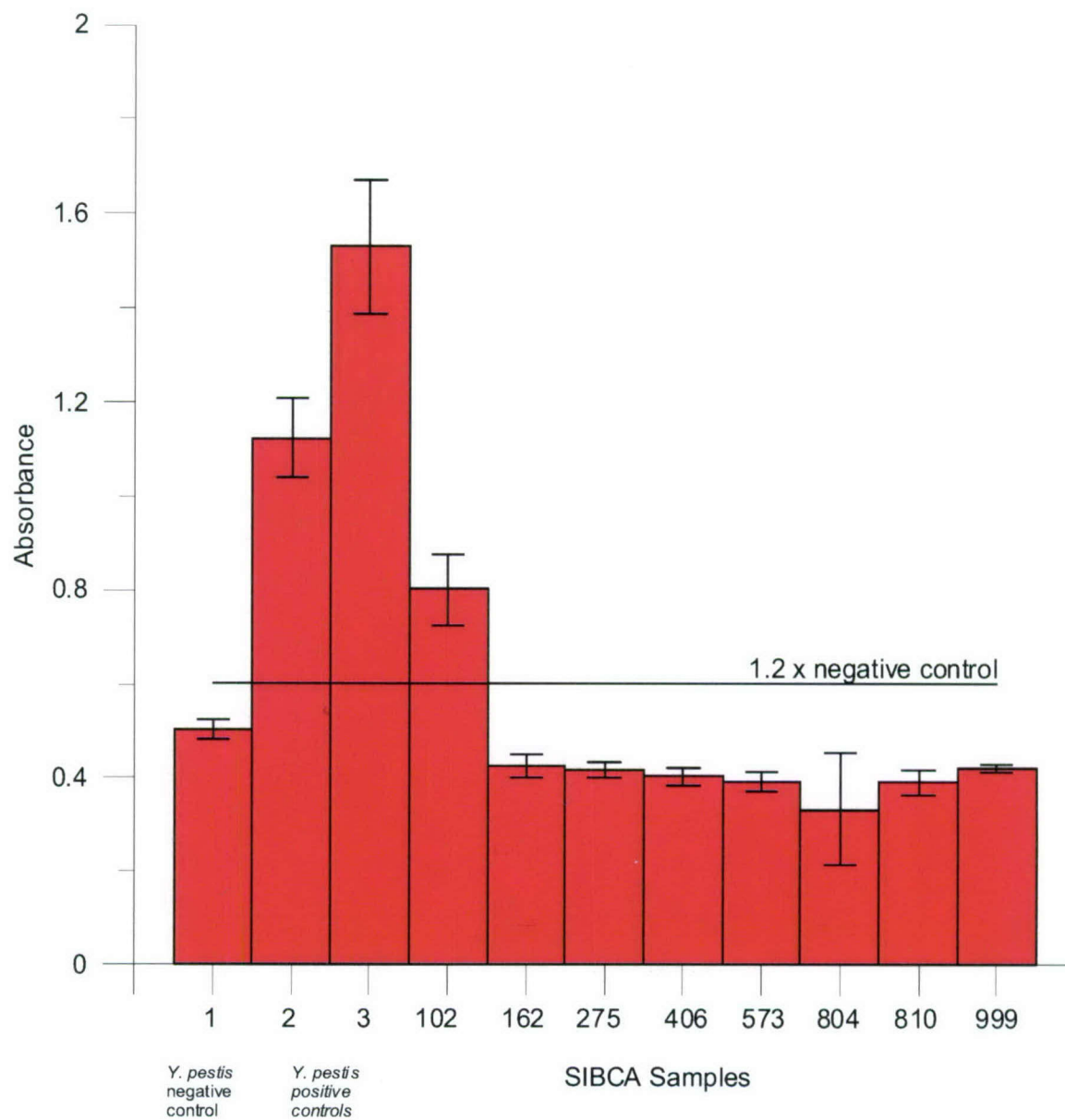


Figure 2. *Y. pestis* ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were 0.9×10^6 and 3.6×10^6 cfu/mL dilutions, respectively. Error bars represent the mean \pm one standard deviation

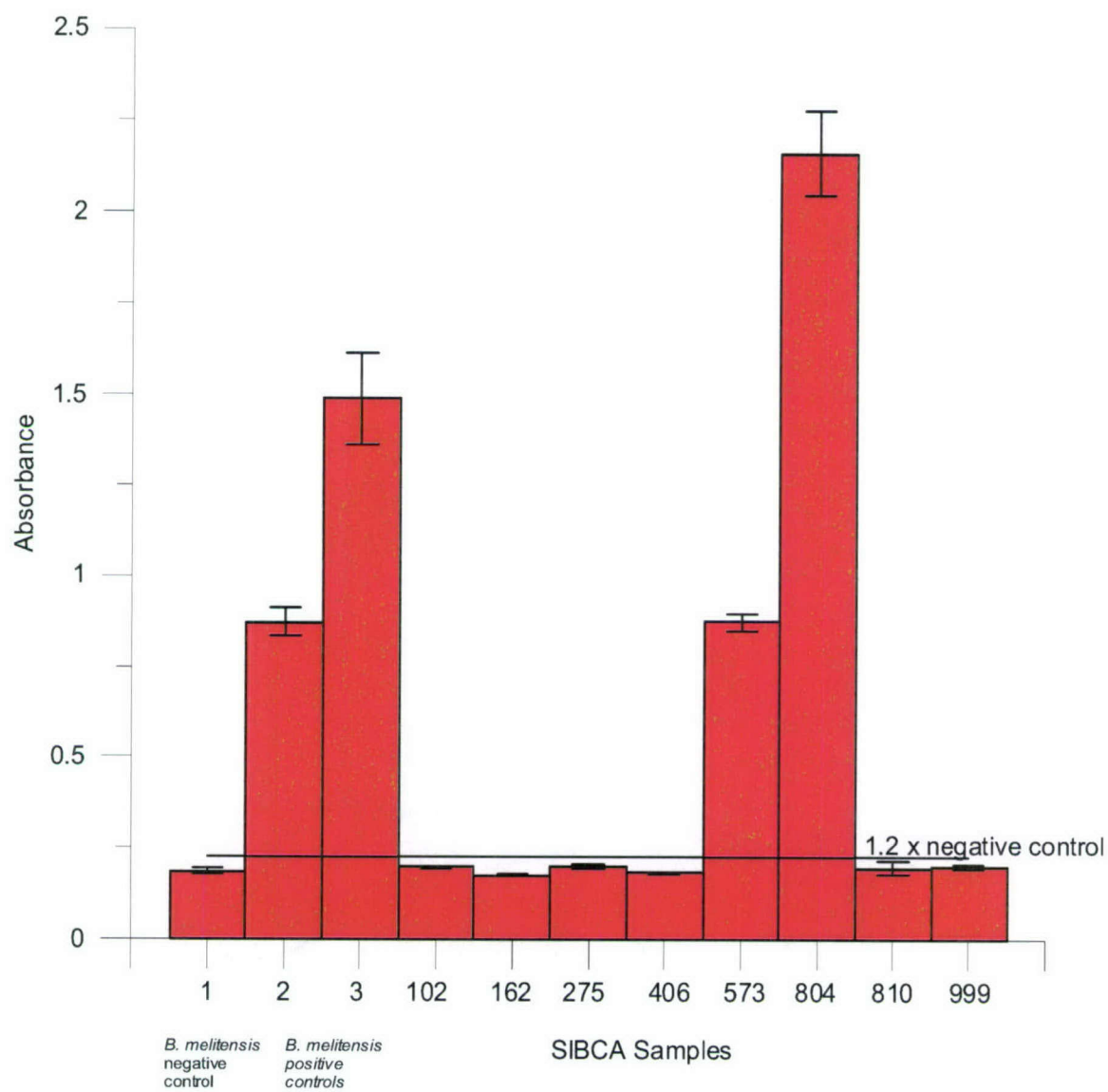


Figure 3. *B. melitensis* ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were 1.1×10^7 and 4.3×10^7 cfu/mL dilutions, respectively. Error bars represent the mean \pm one standard deviation

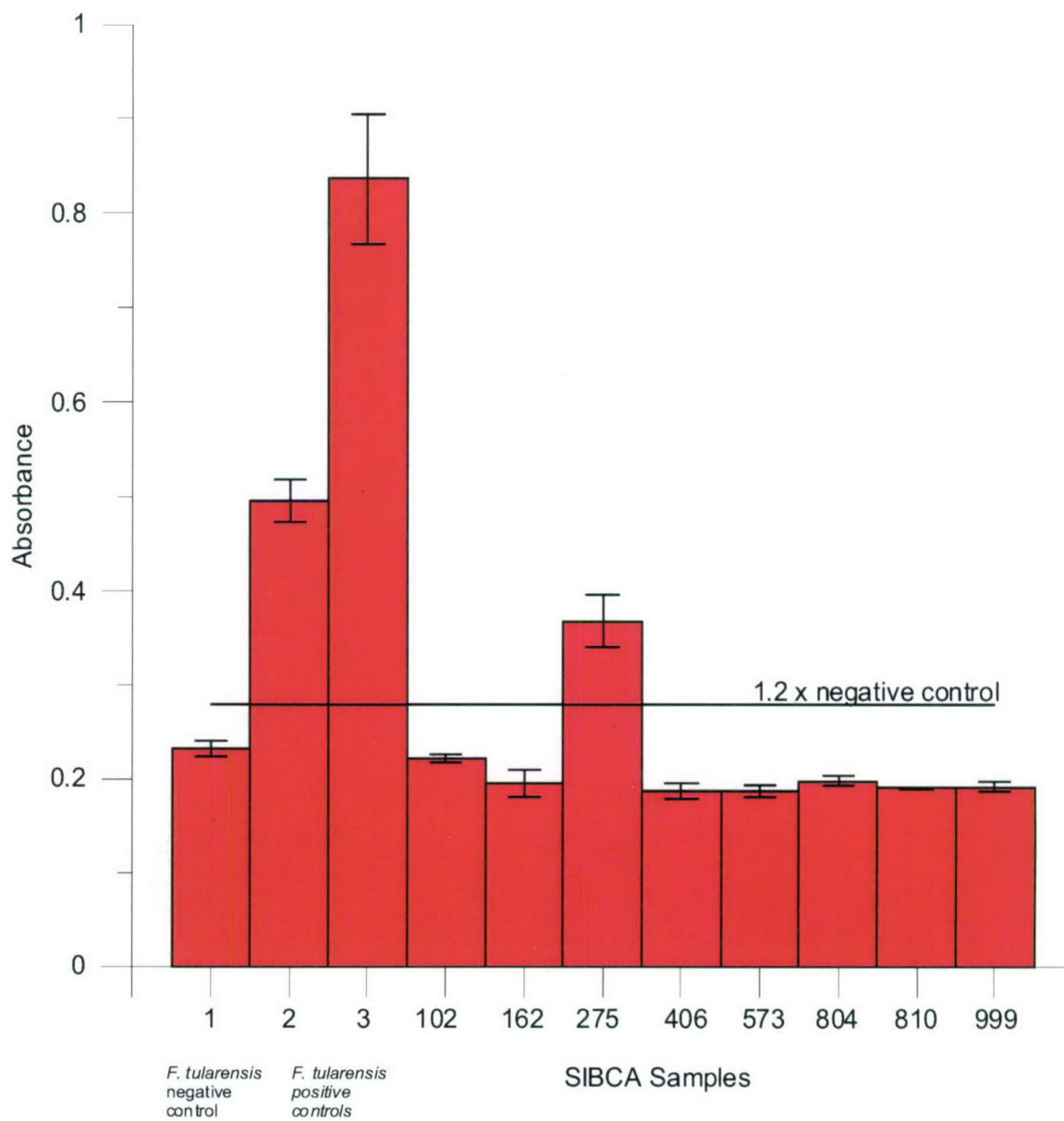


Figure 4. *F. tularensis* ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were 1.95×10^7 and 7.8×10^7 cfu/mL dilutions, respectively. Error bars represent the mean \pm one standard deviation

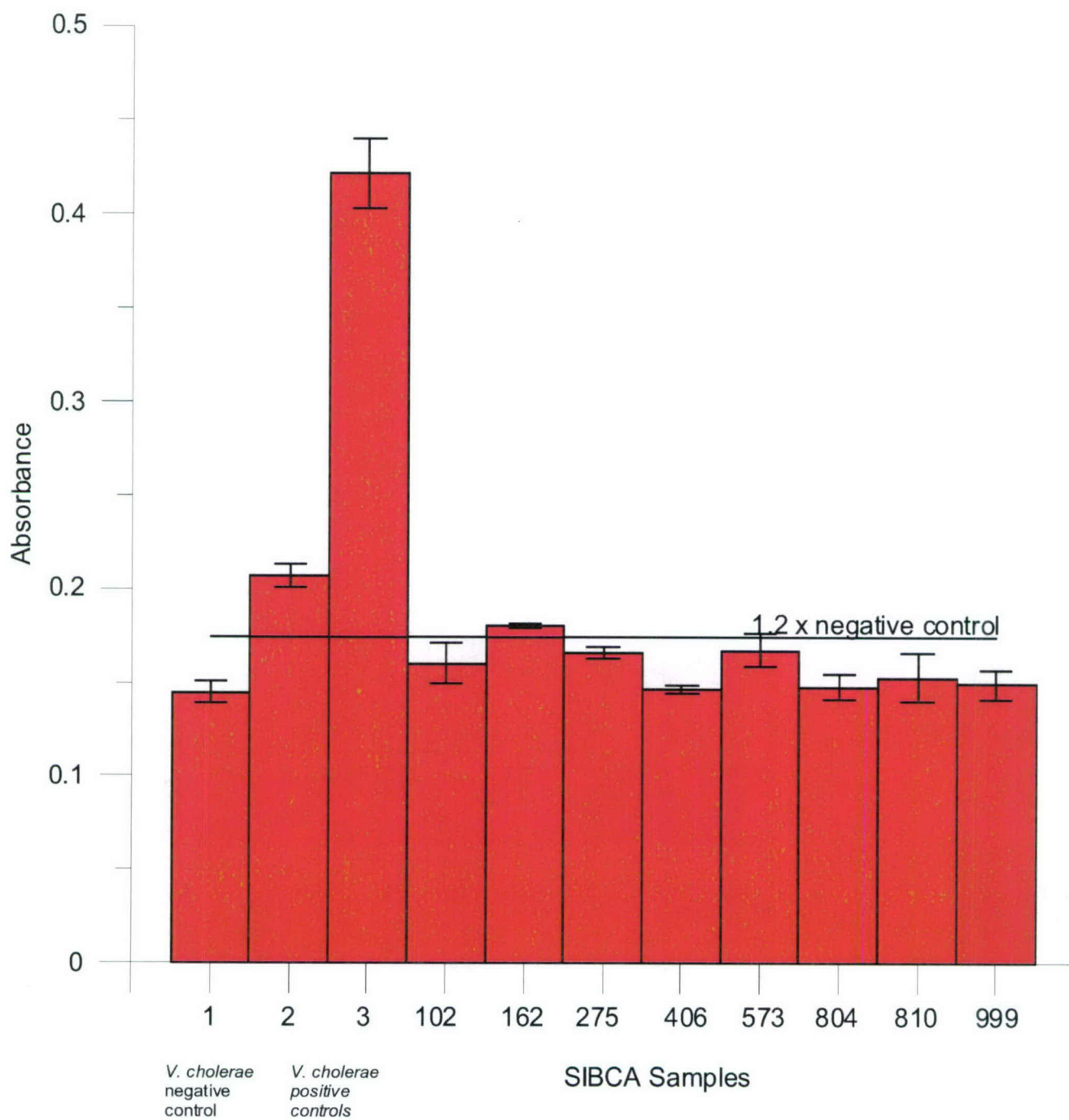


Figure 5. *V. cholerae* ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were $0.5-2.5 \times 10^7$ and $2-10 \times 10^7$ cfu/mL dilutions, respectively. Error bars represent the mean \pm one standard deviation

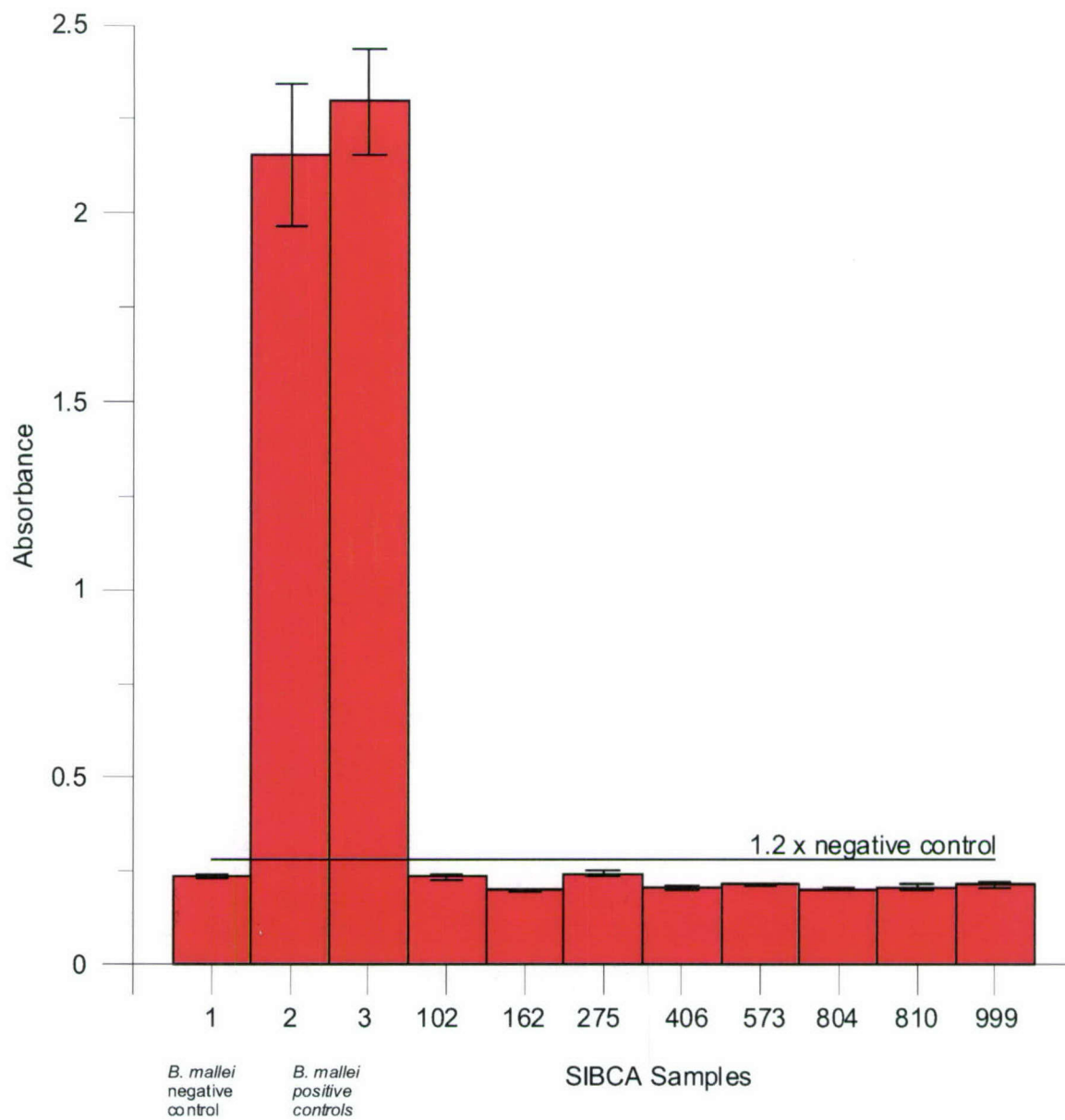


Figure 6. *B. mallei* ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were 1:400 and 1:100 dilutions, respectively. Error bars represent the mean +/- one standard deviation

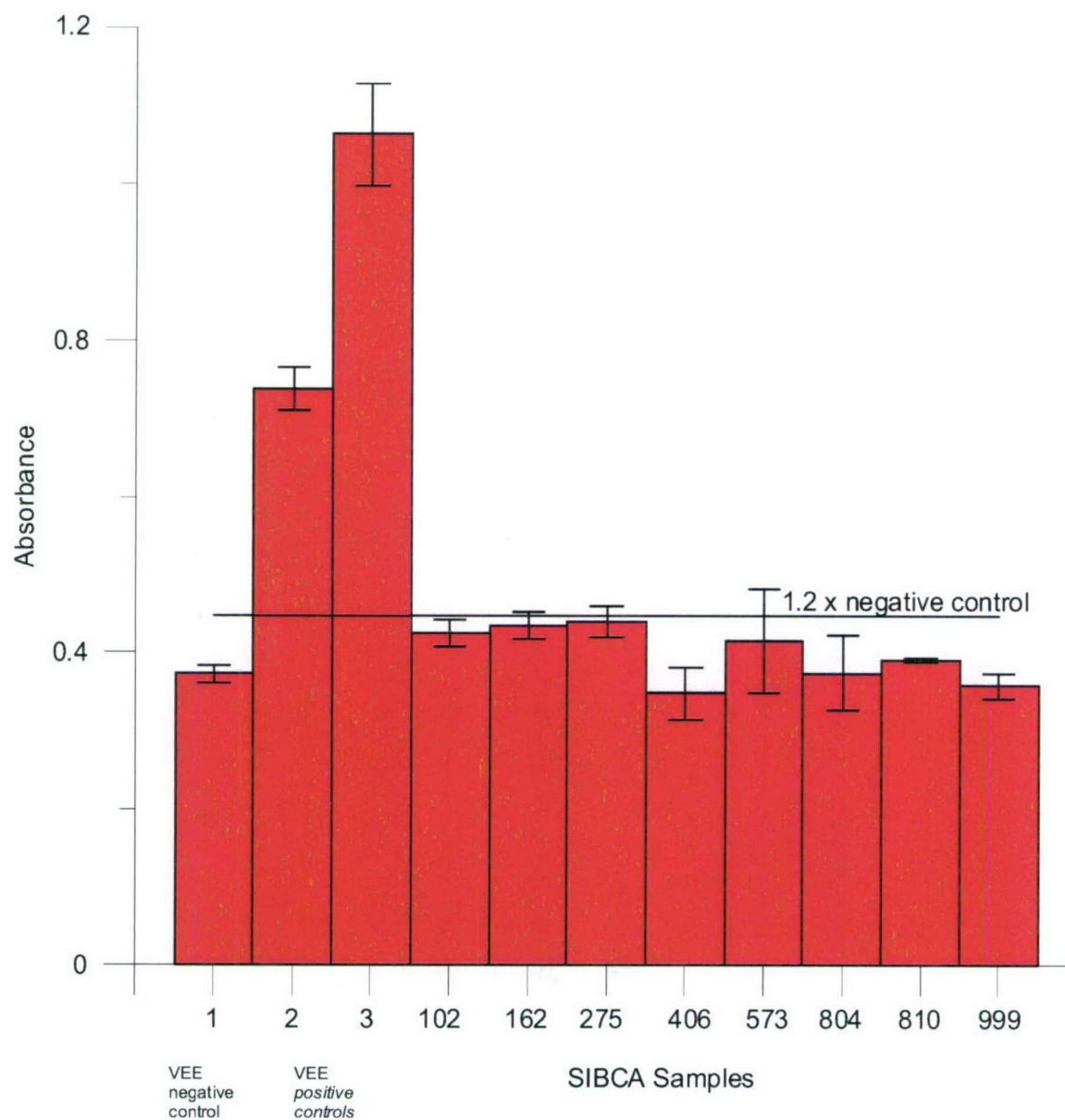


Figure 7. VEE ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were 3 and 12 μ g dilutions, respectively. Error bars represent the mean \pm one standard deviation

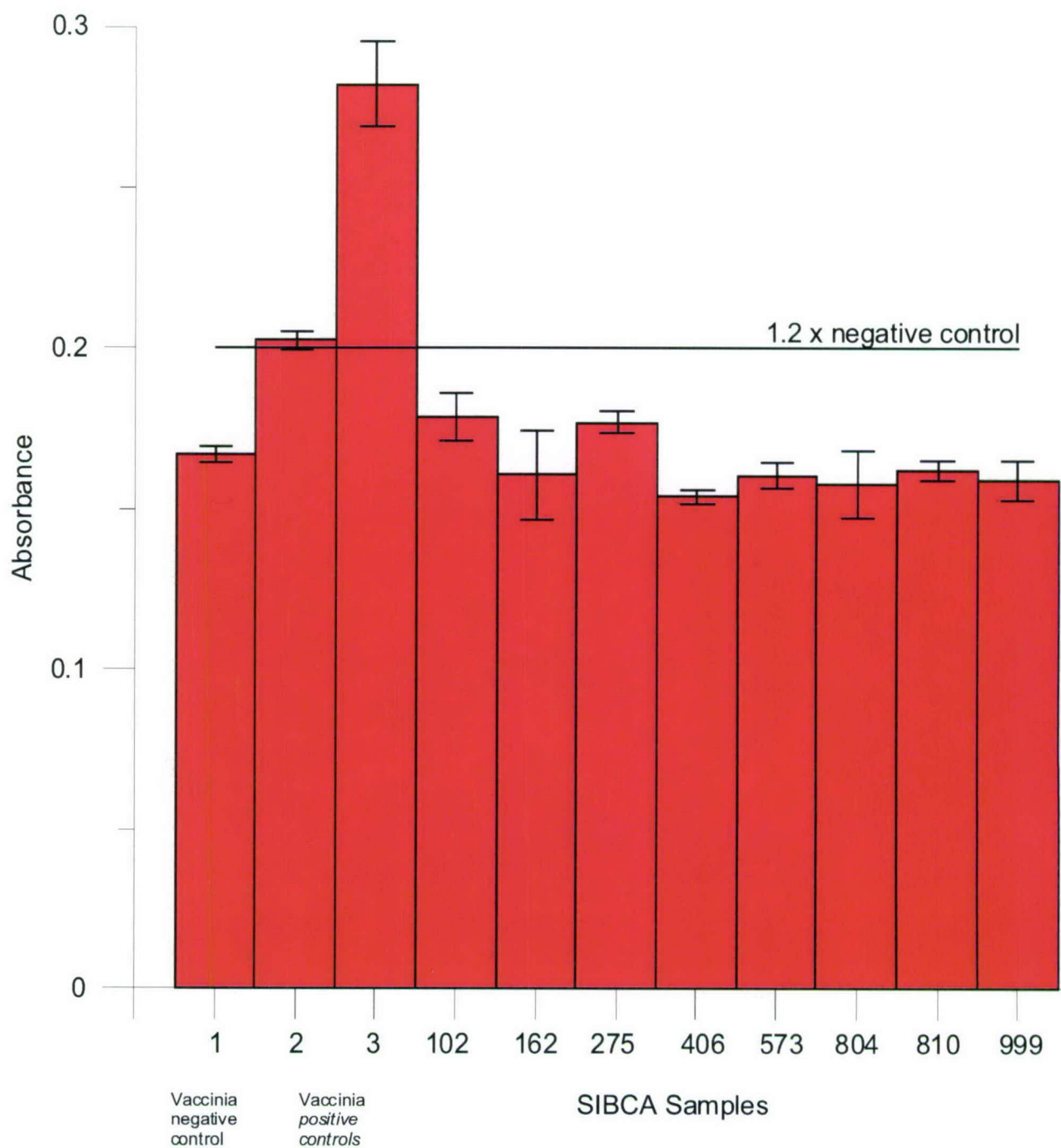


Figure 8. Vaccinia ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were 0.125×10^7 and 0.5×10^7 pfu/mL dilutions, respectively. Error bars represent the mean \pm one standard deviation

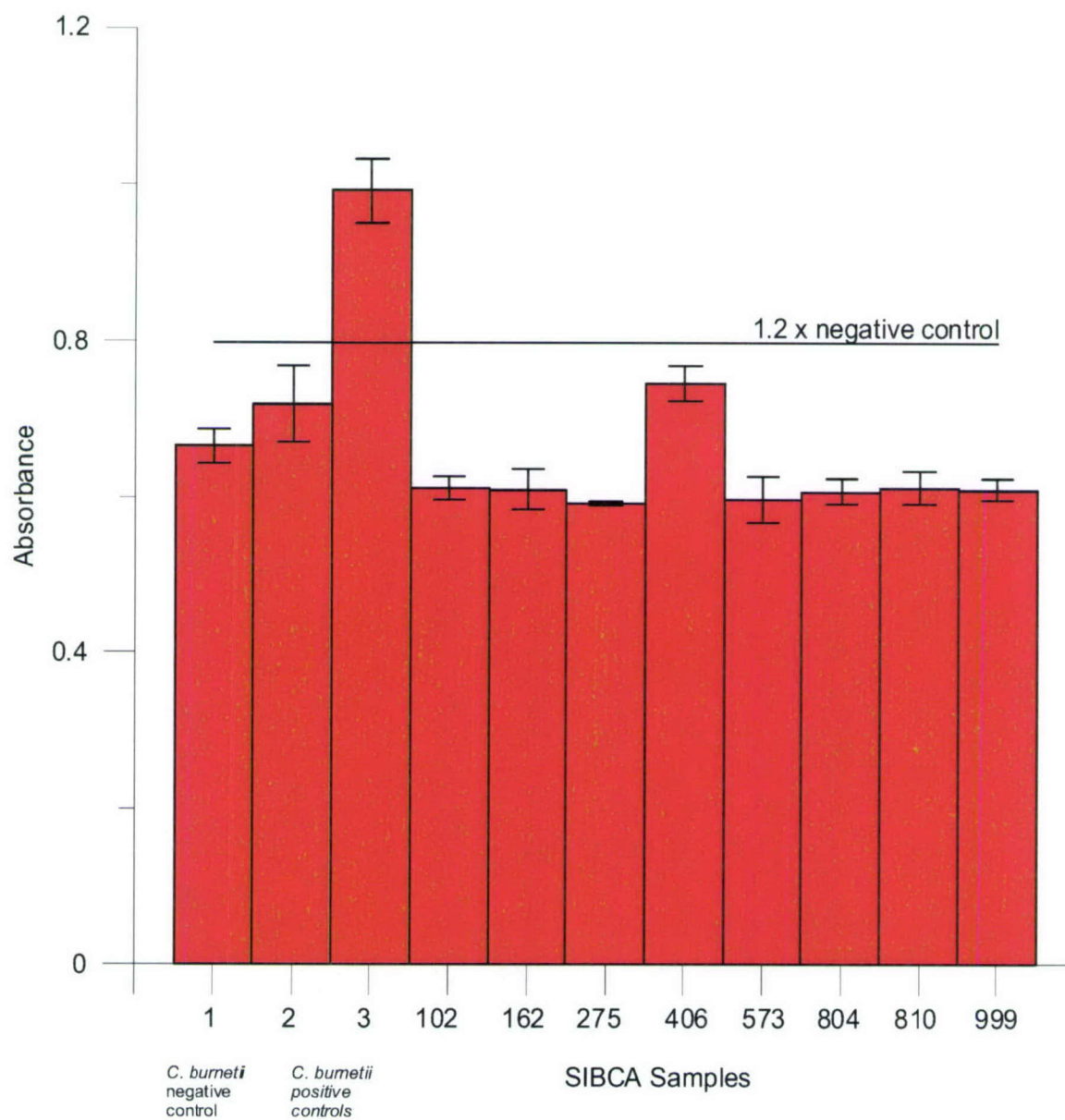


Figure 9. *C. burnetii* ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were 12.5 and 50 μ g dilutions, respectively. Error bars represent the mean +/- one standard deviation

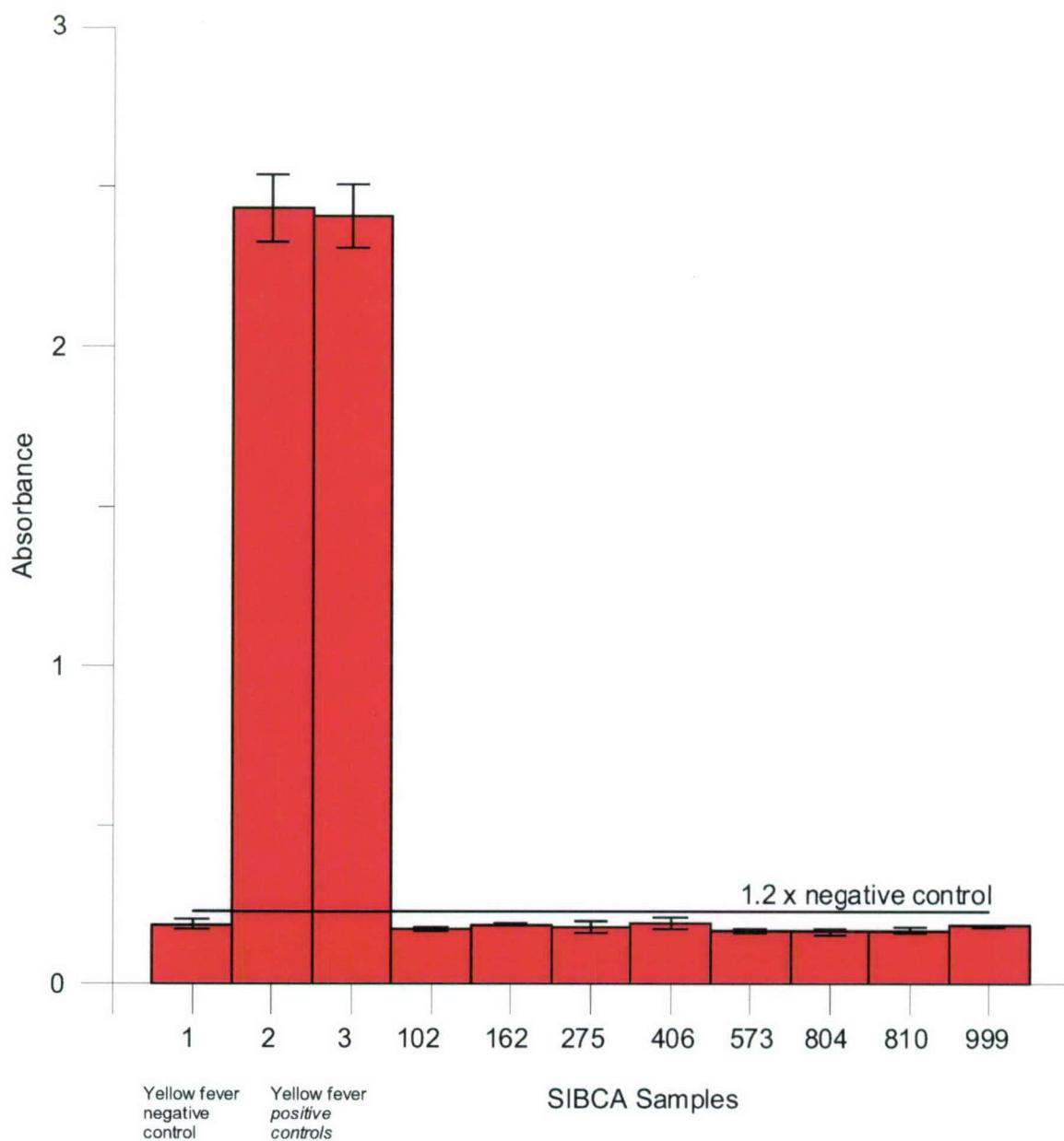


Figure 10. Yellow fever ELISA challenged with SIBCA V sample unknowns. Positive controls 2 and 3 were 1:8 and 1:2 dilutions, respectively. Error bars represent the mean +/- one standard deviation

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In March 2004, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the fifth international training exercise on identification of biological agents. Seventeen NATO/PfP national laboratories participated: Austria, Bulgaria, Canada, Denmark, France, Germany (2 laboratories), Hungary, Italy, The Netherlands, Norway, Poland, Spain, Sweden, Switzerland, the United Kingdom, and the United States. The designated laboratory for Canada was Defence R&D Canada – Suffield (DRDC Suffield). Participant laboratories were sent eight leaves on which sample unknowns had been adsorbed. Participants were advised that samples would contain any one of the following gamma-irradiated organisms: *Bacillus anthracis*, *Yersinia pestis*, *Brucella melitensis*, *Francisella tularensis*, *Vibrio cholerae*, *Burkholderia mallei*, Venezuelan equine encephalitis (VEE) virus, vaccinia virus, *Coxiella burnetii*, or yellow fever virus. An immunological-based technology, the enzyme-linked immunosorbant assay (ELISA), was used at DRDC Suffield for screening of sample unknowns. Antigen capture ELISAs for each of the 10 possible biological agents were used to screen the samples. Five biological agent unknowns were identified by ELISA, three at the species level: *V. cholerae*, *F. tularensis*, and *Y. pestis*, and two at the genus level: both *Brucella spp.* No agents were identified in three additional samples. A comparison of the ELISA results with the identity of organisms in SIBCA sample unknowns, as revealed by Dugway Proving Ground following the exercise, indicated confirmed identification of four of the agents and the sample blank, one unconfirmed identification, and two false negative identifications.

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Enzyme-linked Immunosorbant Assay
ELISA
NATO
SIBCA Exercise V
Biological Agents
Bacillus anthracis
Yersinia pestis
Brucella melitensis
Francisella tularensis
Vibrio cholerae
Burkholderia mallei
Venezuelan equine encephalitis (VEE) virus
Vaccinia virus
Coxiella burnetii
Yellow fever virus

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